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The Hexapeptide Inhibitor of Galβ1,3GalNAc-specific α2,3-Sialyltransferase as a Generic Inhibitor of Sialyltransferases*

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The mammalian Galβ1,3GalNAc-specific α2,3-sialyltransferase (ST3Gal I) was expressed as a secreted glycoprotein in High Five™ (*Trichoplusia ni*) cells. Using this recombinant ST3Gal I, we screened the synthetic hexapeptide combinatorial library to explore a sialyltransferase inhibitor. We found that the hexapeptide, NH_2-GNWWWW, exhibited the most strong inhibition of ST3Gal I among five different hexapeptides that were finally selected. The kinetic analysis of ST3Gal I inhibition demonstrated that this hexapeptide could act as a competitive inhibitor (K_i = 1.1 μM) on CMP-NeuAc binding to the enzyme. Moreover, the hexapeptide was shown to strongly inhibit both *N*-glycan-specific α2,3- and α2,6-sialyltranferase *in vitro*, suggesting that this peptide may inhibit the broad range of sialyltransferases regardless of their linkage specificity. The inhibitory activity *in vivo* was investigated by RCA-I lectin blot analyses and by metabolic D-[6-^{3}H]GlcNH_2 radiolabeling analyses of *N*- and *O*-linked oligosaccharides in Chines hamster ovary cells. Our results demonstrate that the hexapeptide can act as a generic inhibitor of the *N*- and *O*-glycan-specific sialyltransferases in mammalian cells, which results in the significantly reduced NeuAc expression on cellular glycoproteins *in vivo*.

The cell surface oligosaccharides of mammalian cells have been known to function in various cell adhesions and molecular recognition during development, differentiation, and tumor progression (1). In particular, the NeuAcs (sialic acids) have been strongly implicated in tissue inflammation (2, 3) and cancer metastasis (4). Many studies have shown that the increased sialylation is correlated with up-regulation of metastatic potential (5, 6) and also that the oncogenic transformation results in an increased expression of Galβ1,4GlcNAc-specific α2,6-sialyltransferase (7). The sialylations *in vivo* are generally exerted by more than a dozen different sialyltransferases, including glycoprotein-specific α2,3-/α2,6-/α2,8-linkage transferring enzymes and glycolipid-specific α2,3-/α2,8-linkage transferring enzymes (8). Among these, Galβ1,3GalNAc-specific α2,3-sialyltransferase (ST3Gal I)[1] catalyzes the addition of NeuAc from CMP-NeuAc to Galβ1,3GalNAc in an α2,3-specific linkage and completes the chain elongation of core 1 structure in mucin-type *O*-glycosylation. The cDNAs encoding ST3Gal I have been isolated from several species, such as mouse (9), human (10), porcine (11), and chicken (12). Previous studies have shown that ST3Gal I can compete with core 2 β1,6-*N*-acetylglucosaminyltransferase in mucin-type *O*-glycan synthesis (13). Recently, it has been reported that the activity of ST3Gal I is elevated in breast carcinomas, and this elevation of ST3Gal I strongly blocks the conversion of core 1 to core 2 *O*-glycan structure, finally resulting in the shorter length and less complex form of *O*-linked oligosaccharides on MUC1 in breast carcinomas (14). In this regard, the identification of specific inhibitor targeting on sialyltransferases, especially on ST3Gal I, might be a invaluable tool for chemotherapeutic treatment of cancer metastasis and breast carcinoma. Previously, a number of sialyltransferase inhibitors have been reported, such as nucleosides and nucleotide sugar analogues; however, the potency of these inhibitors appears not to be promising for clinical applications (15- 22, 61). In addition, other types of priming inhibitors to block the action of sialyltransferases by competing with endogenous substrates have been described (23). More recently, it has been shown that an endogenous protein inhibitor of Galβ1,4GlcNAc:α2,6-sialyltransferase was isolated from rat serum (24). In the present study, we have identified a hexapeptide inhibitor of sialyltransferase from the synthetic hexapeptide combinatorial library using recombinant ST3Gal I enzyme expressed in insect cells. The kinetic analyses show that the hexapeptide inhibitor affects the K_m value of the donor substrate, CMP-NeuAc, demonstrating that this peptide is functioning as a competitive inhibitor on ST3Gal I in terms of the donor substrate binding. Furthermore, our results demonstrate that the hexapeptide turns out to be generically effective both *in vivo* and *in vitro* to the broad range of sialyltransferases regardless of their linkage specificities.

EXPERIMENTAL PROCEDURES

Materials—Tunicamycin, α2,3-sialyltransferase and α2,6-sialyltransferase were purchased from Roche Molecular Biochemicals. IgG-Sepharose was obtained from Amersham Biosciences. Grace insect culture medium was purchased from Invitrogen. Dowex AG 1-X8 (200–400

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[1] The abbreviations used are: ST3Gal I, Galβ1,3GalNAc:α2,3-sialyltransferase; FITC, fluorescein isothiocyanate; CHO, Chinese hamster ovary.

mesh) was obtained from Bio-Rad. Galβ1,3GalNAc and Me_2SO were purchased from Sigma. Radioactive D-[6-^{3}H]GlcNH$_2$ (14.0 Ci/mmol) and CMP-[9-^{3}H]NeuAc (33.2 Ci/mmol) were purchased from PerkinElmer Life Sciences. Fetal bovine serum was purchased from Hyclone Laboratories Inc. (Logan, UT). The expression vectors in insect cells using the baculovirus system were obtained from Invitrogen. The synthetic hexapeptide combinatorial library was purchased from Postech (Pohang University of Science and Technology, Pohang, Korea).

Cell Cultures and Baculovirus Infection of Insect Cells—CHO-K1 (ATCC) was grown in α-minimal essential medium (Invitrogen) containing 10% heat-inactivated fetal bovine serum supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml). *Spodoptera frugiperda* (Sf9) cells were maintained at 27 °C in Grace medium containing 10% heat-inactivated fetal bovine serum (Hyclone Laboratories) supplemented with antibiotics of penicillin and streptomycin at 50 μg/ml, respectively. The BTI-TN-5B1-4 cells (High Five™ cells; Invitrogen) derived from *Trichoplusia ni* were cultured for recombinant ST3Gal I production at 27 °C in serum-free SF 900 II medium (Invitrogen) supplemented with antibiotics (25). *Autographa californica* nuclear polyhedrosis virus and recombinant baculovirus expressing ST3Gal I were grown and propagated in Sf9 and High Five™ cells.

Construction of the Soluble Form of ST3GalI cDNA in Insect Cell Expression Vector—The soluble form of cDNA containing the catalytic domain of ST3Gal I, which lacks 55 amino acid residues from the NH_2 terminus, was prepared from pUGS α2,3-sialyltransferase (9) by PCR using sense primer (5′-TTCAGAATTCTGCACCTGCAGACACTGC-3′) containing an *Eco*RI site and antisense primer (5′-CATCTCGAGGCATCATCTCCCCTTGAAGAT-3′) containing an *Xho*I site. The amplified 0.95-kb fragment was ligated into the *Eco*RI-*Xho*I site of pcDSA expression vector (26), and the resulting plasmid was designated as pcDSA-ST3Gal I. To construct baculovirus transfer plasmid, pFastBac-ST3Gal I, first, pcDSA-ST3Gal I was partially digested with *Pst*I and blunt-ended with T4 DNA polymerase, and finally, the 1.2-kb DNA fragment containing the IgM signal sequence, the IgG-binding domain of the protein A sequence, and the catalytic domain of ST3Gal I cDNA was isolated by digestion with *Xho*I. Second, the resulting 1.2-kb DNA fragment was introduced into pFastBac-HTa insect cell expression vector (Invitrogen), which was digested with *Rsr*II, blunt-ended with T4 DNA polymerase, and then digested with *Xho*I. The insertion of the DNA fragment in the correct orientation was analyzed by restriction mapping, and the right sequence of the insert junctions was confirmed by DNA sequencing.

Expression and Purification of Soluble ST3Gal I in Insect Cells—The Bac-to-Bac baculovirus expression system based on the site-specific transposition of expression cassette from a donor plasmid into a baculovirus shuttle vector was employed to generate the recombinant virus according to the manufacturer's instruction (Invitrogen). Briefly, pFastBac-ST3Gal I was transformed into *Escherichia coli* DH10Bac-competent cells harboring bacmid DNA (baculovirus whole genome) and transposition helper vector. Then the transposed single white colonies (*lacZ*$^-$) was selected, and recombinant bacmid DNA was isolated by the alkaline lysis method. The recombinant bacmid DNA was transfected into 3 × 10^6 Sf9 cells in a 25-cm^2 T culture flask using Lipofectin™ as recommended by the manufacturer (Invitrogen). After cells and DNA-lipid complex were incubated at 27 °C for 5 h during the transfection, the incubation mixture was replaced with fresh Grace medium. Three days after transfection, the culture supernatant was collected and designated as P1 viral stocks. This P1 was used for generation of the high titer viral stocks by reinfection of Sf9 cells in a 100-mm dish at 50% cell confluence. After 3-day infection, the high titer viral stocks were obtained from culture supernatant, and finally, to produce recombinant ST3Gal I, these high titer viral stocks were used to infect High Five™ cells in the serum-free SF 900 II medium (Invitrogen) at an multiplicity of infection of 10 for 3–5 days. For the purification of ST3Gal I, the High Five™ cell culture supernatant was applied on an IgG-Sepharose column (Amersham Biosciences) at 4 °C overnight, and the column was washed with TBS buffer (10 mM Tris-Cl, pH 7.5, 150 mM NaCl) containing 0.05% Tween 20 followed by elution with 0.1 M glycine buffer (pH 2.6). The fractions containing ST3Gal I were collected and used for the screening of the peptide library. For inhibition of *N*-glycosylation, recombinant ST3Gal I baculovirus-infected High Five™ cells were incubated with tunicamycin at a concentration of 5 μg/ml in serum-free SF 900 II medium. After incubation for 72 h, the medium was collected and applied on a column of IgG-Sepharose. For Western blotting, IgG-Sepharose-eluted fractions were immediately neutralized and subjected to 12% SDS-PAGE under a reducing condition. The gel was electrotransferred to nitrocellulose membrane (Schleicher & Schuell) in Tris/glycine/methanol (25 mM Tris, 192 mM glycine, and 20% methanol), and nitrocellulose membrane was blocked for 2 h at room temperature in TBS buffer containing 5% nonfat dry milk and 0.1% Tween 20. The nitrocellulose membrane was incubated for 1 h with rabbit IgG antibody (rabbit polyclonal anti-protease antibody, prepared in our laboratory, 1:5000 dilution) and overlaid with goat anti-rabbit IgG conjugated with alkaline phosphatase (Pierce). The color was developed with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate in 100 mM $NaHCO_3$ buffer (pH 9.8) containing 5-bromo-4-chloro-3-indolyl phosphate (150 μg/ml) and nitro blue tetrazolium (300 μg/ml) according to the manufacturer's instructions for the alkaline phosphatase color development kit (Bio-Rad).

Measurement of ST3Gal I Sialyltransferase Activity and the Inhibitor Screening of Combinatorial Peptide Library—The assay of ST3Gal I sialyltransferase activity was performed at 37 °C for a 2-h incubation in 50 μl of reaction mixture containing 50 mM sodium cacodylate buffer (pH 7.0), 1 μCi of CMP-[^{3}H]NeuAc, 2 mM $CaCl_2$, 10 mM $MgCl_2$, 13 mM Galβ1,3GalNAc, and 2 μl of recombinant ST3Gal I enzyme. To screen the hexapeptide inhibitor, 2 μl of recombinant ST3Gal I was preincubated with 10 μl of each pool of combinatorial hexapeptide library or solvent (5% Me_2SO) as a control at 37 °C for 0.5 h, and the preincubation mixture was added to the main reaction followed by further incubation for 2 h at 37 °C. After the reaction was completed, the reaction mixture was applied to QAE-Sephadex equilibrated with Tris buffer (pH 9.6) containing 20 mM NaCl, and the flow-through was collected. The radioactivity contained in the flow-through was measured in a liquid scintillation counter (Beckman), and then the flow-through was applied on a column of Bio-Gel P-4 (1 × 100 cm) equilibrated with 100 mM sodium bicarbonate buffer (pH 7.6) to separate the reaction product from free [^{3}H]NeuAc.

Comparison of ST3Gal I Inhibitory Activity of Five Hexapeptides and Kinetic Analysis of ST3Gal I Inhibition—The peptide sequences were identified from the initial screening of 114 pools of combinatorial peptide library, and the possible five different hexapeptide sequences (P1–P5) were prepared by solid phase chemical synthesis using a semiautomated synthesizer (Peptron Inc., Taejon, South Korea). The inhibitory activity of five hexapeptides was compared by incubation at 37 °C for 2 h in 50 μl of main reaction mixture containing 50 mM sodium cacodylate buffer (pH 7.0), 1 μCi of CMP-[^{3}H]NeuAc, 2 mM $CaCl_2$, 10 mM $MgCl_2$, 13 mM Galβ1,3GalNAc, and 2.5 μl of recombinant enzyme preincubated with increasing concentrations of each peptide (P1–P5), respectively, or solvent (5% Me_2SO) as a control at 37 °C for 0.5 h. To obtain the apparent K_m and V_{max}, the kinetic mode of ST3Gal I was monitored with varying concentrations of CMP-NeuAc and Galβ1,3GalNAc. In the case of varying concentrations of CMP-NeuAc, 2.5 μl of recombinant ST3Gal I was preincubated with a fixed concentration of P5 hexapeptide and added to the main reaction mixture containing 50 mM sodium cacodylate buffer (pH 7.0), various concentrations of cold CMP-NeuAc (from 10 μM to 2 mM) with 5 × 10^4 cpm of CMP-[^{3}H]NeuAc, 2 mM $CaCl_2$, 10 mM $MgCl_2$, and 1 mM Galβ1,3GalNAc, followed by further incubation for 1 h at 37 °C. In case of varying concentrations of Galβ1,3GalNAc, 2.5 μl of recombinant ST3Gal I was preincubated with hexapeptide P5 and added to the main reaction mixture containing 50 mM sodium cacodylate buffer (pH 7.0), various concentrations of Galβ1,3GalNAc (from 10 μM to 2 mM), 2 mM $CaCl_2$, 10 mM $MgCl_2$, and 100 μM CMP-NeuAc with 5 × 10^4 cpm of CMP-[^{3}H]NeuAc. The reaction was stopped with ice-cold distilled H_2O and applied to QAE-Sephadex in 2 mM Tris buffer (pH 9.6) containing 20 mM NaCl. The flow-through was collected and applied to Bio-Gel P-4 column, and the reaction product was fractionated. The radioactivity contained in the product was determined by measuring in a Beckman liquid scintillation counter.

Inhibition of Other Sialyltransferase Activities in Vitro—10 milliunits of *N*-glycan-specific α2,3- or α2,6-sialyltransferase preincubated with either 100 μM of peptide inhibitor P5 or Me_2SO solvent as a control, respectively, was mixed with a 50-μl reaction mixture containing 50 mM sodium cacodylate (pH 7.0), 1 μCi of CMP-[^{3}H]NeuAc, 2 mM $CaCl_2$, 10 mM $MgCl_2$, and 10 mM Galβ1,4GlcNAc and incubated at 37 °C for 2 h. The reaction was stopped, and the sample was passed over a column of QAE-Sephadex. Finally, the reaction product was separated from free [^{3}H]NeuAc by Bio-Gel P-4 column chromatography.

Inhibition of NeuAc Expression in Vivo—The CHO cells cultured in 60-mm dish containing 2 ml of complex culture medium were treated with a dose-dependent increase of P5 hexapeptide or FITC-P5 inhibitor (10, 30, and 100 μM) for 72 h. During this period, the medium containing an inhibitor peptide or a control peptide was freshly changed at 24 h of incubation to maintain the peptide concentration. Cells were washed with cold 1× PBS buffer (10 mM KH_2PO_4 and 150 mM NaCl, pH 7.2), and cell lysates were prepared by mild sonication in PBS buffer con-

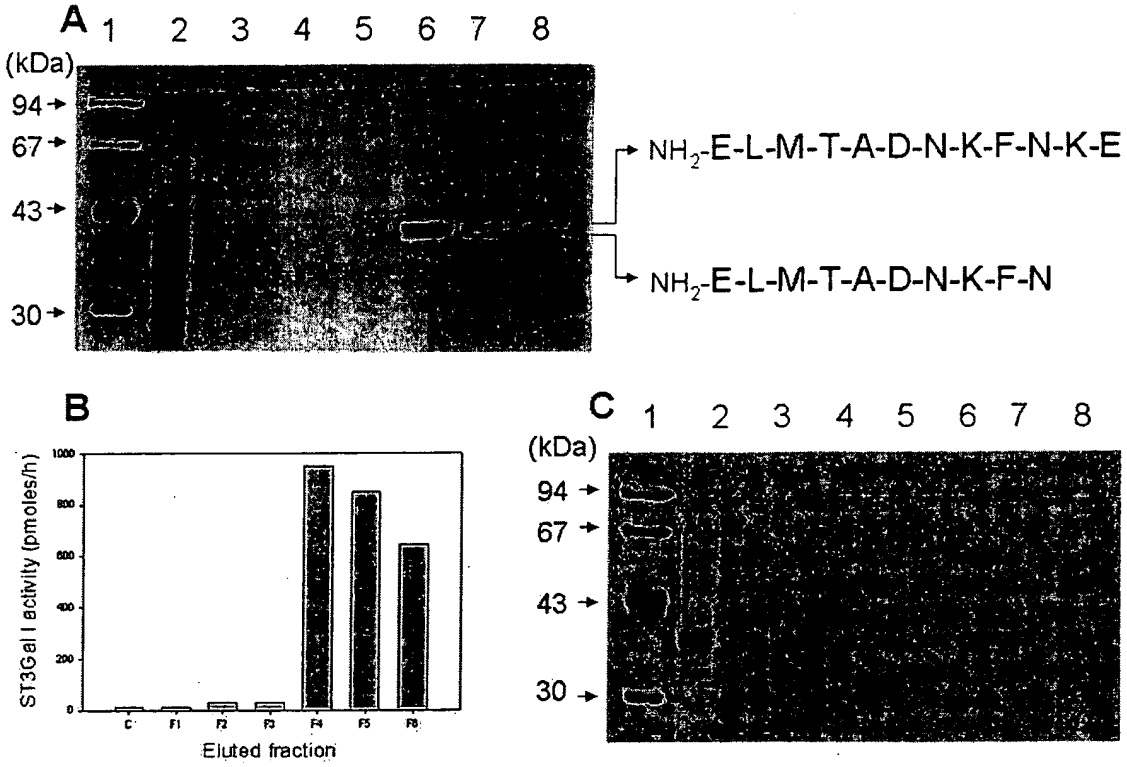

FIG. 1. **Recombinant ST3Gal I is secreted as two different molecular mass polypeptides in High Five™ insect cells.** *A*, the High Five™ cells infected with ST3Gal I recombinant baculovirus were cultured for 72 h, and the medium was collected and applied on a column of IgG-Sepharose. The bound ST3Gal I was eluted with low pH buffer as described under "Experimental Procedures." The flow-through (*lane 2*) and eluted fractions from the IgG-Sepharose column, *F1–F6* (*lanes 3–8*) were analyzed by SDS-PAGE followed by Coomassie staining. The recombinant ST3Gal I eluted as two different molecular masses was identified by a direct microsequencing of NH_2-terminal amino acid sequence as the *arrows* indicate. *B*, the ST3Gal I activities of eluted fraction 1 (*F1*) to fraction 6 (*F6*) were determined (*bar F1* to *bar F6*), and control cell medium with no ST3Gal I activity was assayed as a control (*bar c*). *C*, the flow-through and eluted fractions 1 (*F1*) to fraction 6 (*F6*) from IgG-Sepharose applied by tunicamycin-treated cell medium (*lanes 3–8*) were analyzed by SDS-PAGE followed by Coomassie staining. The molecular weight marker was loaded on the gel (*lane 1*).

taining 1% Nonidet P-40 and protease inhibitor mixtures. The cell lysates were subjected to SDS-PAGE (27) followed by Western blot analysis. For lectin blot analysis, transferred nitrocellulose membrane was incubated with 5% bovine serum albumin at 4 °C overnight for blocking and overlaid with 6 μg/ml biotinylated RCA-I lectin (*Ricinus communis* agglutinin 120; Sigma) followed by streptavidin-peroxidase (Roche Molecular Biochemicals; 1:4000 dilution). The signal was developed on Eastman Kodak Co. BioMax-MR-1 film with ECL reagents (Amersham Biosciences). To analyze the oligosaccharides directly, CHO cells were treated with peptide and Chariot™, a specific liposome system commercially available from Active Motif (Carlsbad, CA). The Chariot™ was mixed with 50 μM P5 hexapeptide (NH_2-GNWWWW), control hexapeptide (NH_2-WRGGSG), and Me_2SO only and used to treat CHO cells for 2 h according to the manufacturer's recommended procedure. Subsequently, cells were metabolically D-[6-^{3}H]GlcNH_2-radiolabeled for 8 h in complex medium containing the final concentration of 200 μCi/ml of radioactive sugar, and the labeled cells were resuspended in PBS buffer followed by gentle sonication on ice. The sonicated supernatants were mixed with methanol/chloroform (1:1, v/v), and lipids were extracted by vortexing. The white pellets in boundary region between the upper and lower layer were collected and washed with ice-cold 90% aceton. The aceton-washed total glycoproteins were directly treated in 50 mM NaOH containing 1 M $NaBH_4$ at 45 °C for 16 h to allow β-elimination (28), and the released *O*-linked oligosaccharides were analyzed by descending paper chromatography as described previously (29). For the analysis of *N*-linked oligosaccharides, the treatment of *N*-glycanase was performed as previously described (30). Briefly, the labeled cells were sonicated in 20 mM Tris buffer (pH 7.4) for three times, and SDS was added for a final concentration of 0.2%. The total cell lysates was boiled at 100 °C for 10 min and centrifuged at 15,000 × *g* for 15 min. The clear lysates was applied to Sephadex G-50 (1 × 90 cm), and the column was eluted with 20 mM Tris (pH 7.4) containing 0.2% SDS. The void fractions containing glycoproteins were pooled and precipitated by ice-cold 90% aceton. The precipitates was redissolved in 50 mM sodium phosphate (pH 7.4) containing 0.5% SDS, 50 mM β-mercaptoethanol and boiled for 5 min followed by the addition of 5 units of *N*-glycanase according to manufacturer's recommendation (Roche Molecular Biochemicals). The reaction mixture was incubated at 37 °C overnight, and the reaction was stopped by boiling for 5 min followed by rechromatography on Sephadex G-50 column. Fractionated samples containing the released oligosaccharides were pooled, KCl was added to remove residual SDS and further desalted by Sephadex G-10 chromatography. Finally, *N*-linked oligosaccharides were analyzed by QAE-Sephadex in 2 mM Tris buffer (pH 9.0), and the charged glycans were eluted with increasing concentrations of 20, 70, 140, and 200 mM NaCl, respectively. For mild acid treatment to prepare desialylated *N*- and *O*-linked oligosaccharides, the released glycans were treated with 2 N acetic acid at 100 °C for 1 h as described previously (29).

RESULTS

Expression and Purification of Recombinant ST3Gal I as a Functionally Active Enzyme in Insect Cells—We expressed the ST3Gal I in recombinant baculovirus-infected High Five™ cells as a soluble and secreted enzyme fused with IgG-binding domain. The ST3Gal I was purified in one step by an IgG-Sepharose column from culture supernatants, and bound ST3Gal I was eluted with 0.1 M glycine buffer (pH 2.6) followed by an immediate neutralization. The eluted fractions were pooled and analyzed by SDS-PAGE and Coomassie staining (Fig. 1). The soluble form of recombinant ST3Gal I was secreted as two different molecular mass forms corresponding to 41 kDa and 40 kDa (Fig. 1*A*). To investigate the identity of these polypeptides, first, NH_2-terminal amino acid sequence was directly determined by protein microsequencing, and second, ST3Gal I sialyltransferase activities of the eluted fractions

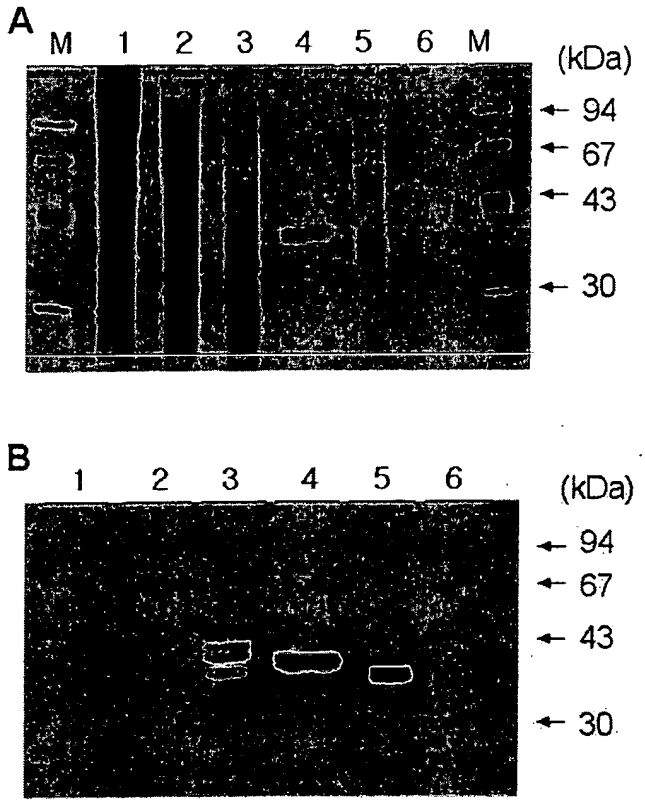

FIG. 2. **Analysis of the cell-associated ST3Gal I expressed in High Five™ insect cells.** *A*, the cellular proteins prepared from High Five™ cells were subjected to SDS-PAGE followed by Coomassie staining. Intact High Five™ cell lysates (*lane 1*), wild-type *A. californica* nuclear polyhedrosis virus baculovirus-infected cell lysates (*lane 2*), ST3Gal I recombinant baculovirus-infected cell lysates (*lane 3*), the ST3Gal I in F4 fraction eluted from the IgG-Sepharose column (*lane 4*), ST3Gal I recombinant baculovirus-infected cell lysates in the presence of tunicamycin (*lane 5*), and the eluted F4 fraction from the IgG-Sepharose column applied by tunicamycin-treated culture medium (*lane 6*) were loaded on the gel, respectively. *B*, the same gel was analyzed by Western blotting using rabbit IgG as a primary antibody as described under "Experimental Procedures." The molecular weight marker was loaded on the gel (*lane M*).

were measured. As shown in Fig. 1*A*, the NH_2-terminal amino acid sequences of two polypeptides were identical and found to be matched with protein A sequence of IgG-binding domain just after cleavage of IgM signal sequence (26). Furthermore, ST3Gal I sialyltransferase activity was correlated with the protein intensity in Coomassie staining (Fig. 1*B*). These results suggest that the signal sequence of mouse IgM can be correctly cleaved in High Five™ cells and that the 1-kDa difference of molecular mass could be caused by either glycosylation or other protein modifications.

The Expression of Recombinant ST3Gal I after Tunicamycin Treatment—To test whether the 1-kDa difference of molecular mass is caused by *N*-glycosylation, High Five™ cells expressing ST3Gal I were incubated with 5 μg/ml tunicamycin to block *N*-glycosylation. The culture medium was applied on a column of IgG-Sepharose, and the bound ST3Gal I was eluted by low pH buffer. But no protein was eluted out of the IgG-Sepharose column (Fig. 1*C*), indicating that ST3Gal I deficient in *N*-linked oligosaccharides seemed not to be secreted into medium. We examined the presence of ST3Gal I in the culture medium by trichloroacetic acid precipitation of culture supernatants and Western blotting; however, ST3Gal I was not contained in tunicamycin-treated medium (data not shown). We further examined whether ST3Gal I is present in cell lysates by SDS-PAGE (Fig. 2*A*) and Western blot analysis (Fig. 2*B*). When cells were treated with tunicamycin, ST3Gal I was present exclusively in cell lysates (Fig. 2*B*, *lanes 5* and *6*), indicating that *N*-glycosylation of ST3Gal I might be crucial for protein secretion in High Five™ cells. Moreover, ST3Gal I after tunicamycin treatment still gave rise to two different molecular masses with the same 1-kDa difference (Fig. 2*B*, *lane 5*), indicating that the difference of molecular mass does not result from *N*-glycosylation. It should be mentioned that several other molecular weight forms of ST3Gal I were also detected on Western blotting of cell lysates (Fig. 2*B*, *lane 3*), indicating that some unprocessed and/or intermediately glycosylated forms of ST3Gal I may reside inside cells.

Screening of Hexapeptide Library Pools for Identifying the Peptide Inhibitor of Recombinant ST3Gal I—The screening of a combinatorial hexapeptide library containing 114 peptide pools allowed us to determine the most effective amino acid residues that can be positioned in a hexapeptide sequence. The screening of the peptide library was performed as shown in Fig. 3. Screening of the first pool containing the NH_2-*XXXXX*-X_1 peptide mixtures showed that NH_2-*XXXXX*-W was found to most strongly inhibit ST3Gal I activity (Fig. 3*A*). The other three amino acid residues of glycine, asparagine, and arginine at the X_1 position appear to be less active than tryptophan. The screening of the second, third, and fourth pool of the peptide library also revealed that the tryptophan residue was the highest inhibitory amino acid at the X_2, X_3, and X_4 positions (Fig. 3, *B–D*). In the case of the fifth pool of NH_2-*XX*$_5$-*XXXX*, asparagine, arginine, tryptophan, and tyrosine were identified as strongly inhibitory amino acid residues, and among these, asparagine showed the highest inhibitory activity at the X_5 position (Fig. 3*E*). Finally, the sixth position was identified as glycine, which was the most inhibitory element among 19 amino acids (Fig. 3*F*). Based on these results, the five possible hexapeptides were synthesized as follows: the first position from the NH_2 terminus, Gly; the second position, Asn and Trp; the third position, Trp and Arg; the fourth position, Trp; the fifth position, Trp; and the sixth position, Trp and Arg. The amino acid sequences of the five hexapeptides finally chosen are listed in Fig. 4*A*. The relative inhibitory activity of each peptide was tested, showing that the P5 hexapeptide sequence, NH_2-GNWWWW, was found to most strongly inhibit ST3Gal I activity (Fig. 4*B*).

Kinetic Analysis of ST3Gal I Inhibition by P5 Hexapeptide—The NH_2-GNWWWW designated as P5 hexapeptide was utilized to study the kinetic mode of ST3Gal I inhibition. In kinetic analysis, first, recombinant ST3Gal I and radioactive CMP-[^{3}H]NeuAc are incubated with varying concentrations of non-radioactive CMP-NeuAc under a fixed concentration of Galβ1,3GalNAc substrate (Fig. 5*A*). Second, enzyme and CMP-[^{3}H]NeuAc are incubated with varying concentrations of Galβ1,3GalNAc under a fixed concentration of CMP-NeuAc (Fig. 5*B*). Kinetic assays were demonstrated to be linear with respect to incubation time and amount of recombinant ST3Gal I (data not shown). In Fig. 5*A*, the apparent K_m and V_{max} of ST3Gal I enzyme for CMP-NeuAc were determined to be 38 μM and 650 pmol/h using 2.5 μl of purified ST3Gal I, respectively. In the presence of peptide inhibitor, K_m was changed without alteration of V_{max}, suggesting that P5 hexapeptide was acting as a competitive inhibitor (K_i = 1.1 μM) of ST3Gal I in terms of CMP-NeuAc binding (Fig. 5*A*). In Fig. 5*B*, the apparent K_m and V_{max} of ST3Gal I enzyme for Galβ1,3GalNAc were determined to be 268 μM and V_{max} of 1740 pmol/h using 2.5 μl of purified ST3Gal I. In the presence of peptide inhibitor, V_{max} was changed without alteration of K_m, indicating that P5 hexapeptide was acting as a noncompetitive inhibitor (K_i = 8.8 μM) of

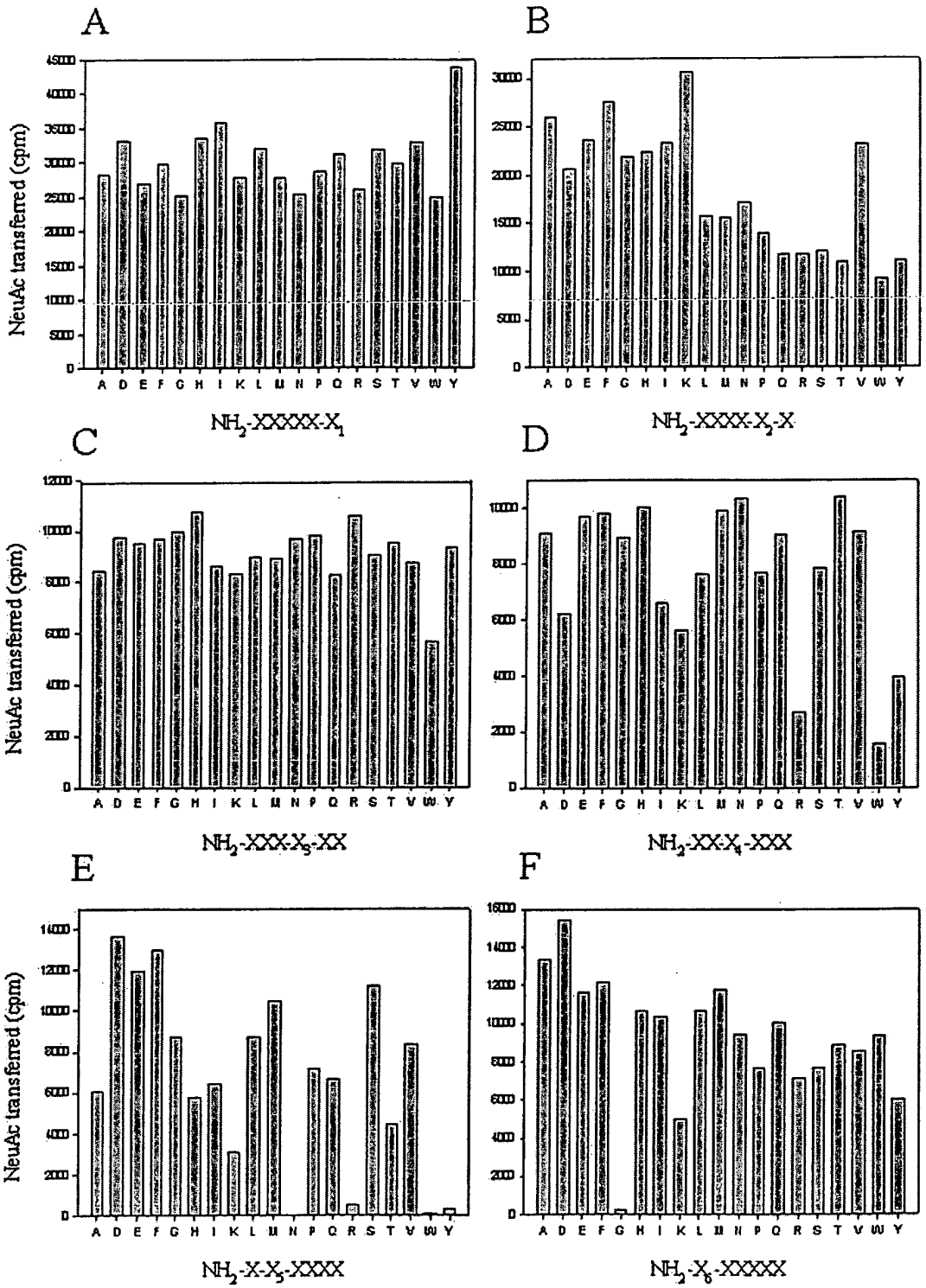

FIG. 3. **Screening of hexapeptide library pool for the identification of ST3Gal I inhibitor.** *A–F*, six hexapeptide libraries containing total 114 peptide pools were screened for ST3Gal I inhibitory activity to define the hexapeptide sequence. One hexapeptide library consisted of 19 different peptide pools. Each position (X_1 to X_6) of the hexapeptide sequence (NH_2-X_6-*XXXX*-X_1) is occupied by one of the 19 L-amino acids except for the cysteine residue. The 19 peptide pools (*lanes A–Y*) of each hexapeptide library were preincubated with recombinant ST3Gal I, respectively, and enzyme activities were assayed using CMP-[^{3}H]NeuAc as described under "Experimental Procedures." The *bar* in each *panel* represents the amount of transferred NeuAc by ST3Gal I activity in the presence of each of the 19 peptide pools, respectively.

ST3Gal I in terms of Galβ1,3GalNAc binding. Together, these results demonstrate that P5 hexapeptide can competitively inhibit CMP-NeuAc binding to ST3Gal I, whereas this peptide appears not to affect Galβ1,3GalNAc binding to ST3Gal I.

Inhibition of Other Sialyltransferase Activities by P5 Hexapeptide—Based on these results, it is possible to deduce that P5 hexapeptide may inhibit other types of sialyltransferases because all sialyltransferases known thus far utilize CMP-NeuAc as a common donor substrate. Therefore, we tested whether P5 could inhibit the activities of other sialyl-

A

Peptides	Amino acid sequence
P1	NH_2-GWWWWWW
P2	NH_2-GWRWWW
P3	NH_2-GWRWWR
P4	NH_2-GWWWWR
P5	NH_2-GNWWWWW

B

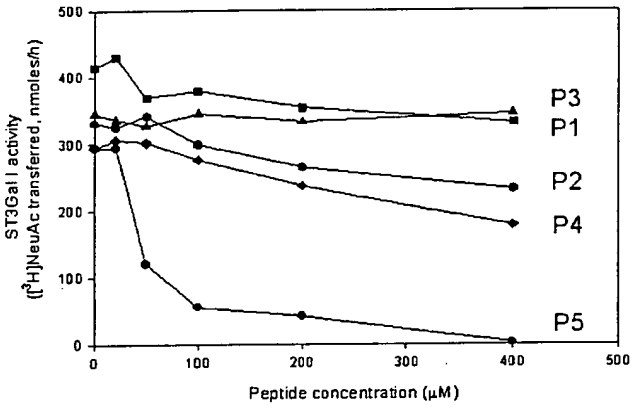

FIG. 4. **Comparison of ST3Gal I-inhibitory activity of five individual hexapeptides.** *A*, the five individual hexapeptides were synthesized and each peptide was designated as P1–P5. The peptide sequences are represented with single-letter amino acid codes. *B*, the comparative analysis of ST3Gal I inhibition was performed using CMP-[^{3}H]NeuAc at varying concentrations (0–400 μM) of P1–P5.

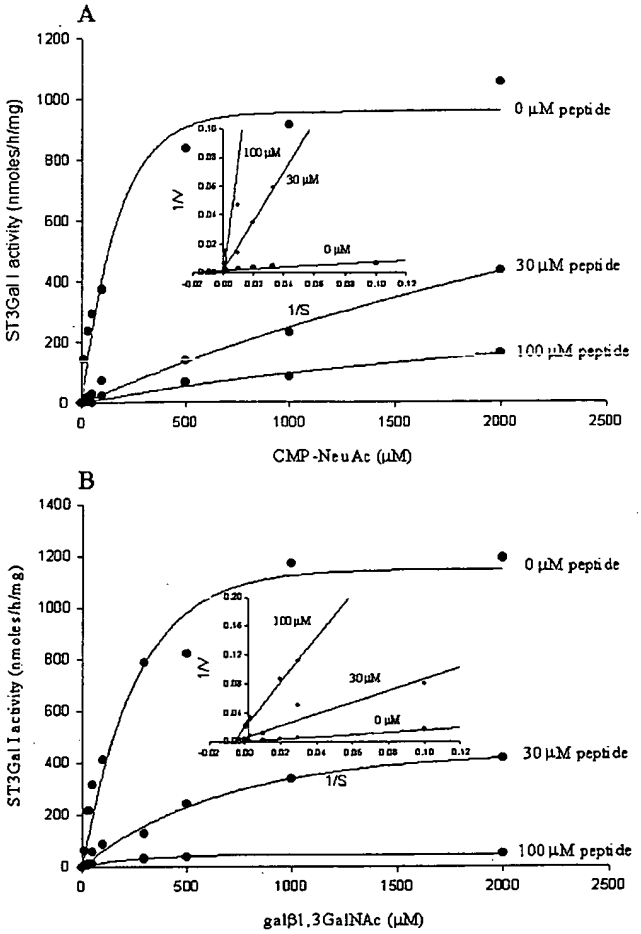

FIG. 5. **Kinetic analysis of ST3Gal I inhibition by P5 hexapeptide.** *A*, the Michaelis-Menten plot of ST3Gal I activity *versus* CMP-NeuAc concentration was drawn in the presence of increasing concentrations of P5 inhibitor (0–100 μM). *B*, the same plot of ST3Gal I activity *versus* Galβ1,3GalNAc concentration was drawn in the presence of increasing concentrations of P5 inhibitor (0–100 μM). The activity of ST3Gal I was measured under the standard condition as described under "Experimental Procedures," and the effect of P5 peptide inhibitor on ST3Gal I was shown in *insets* for the determination of V_{max} and K_m.

transferases such as *N*-glycan-transferring α2,3- and α2,6-sialyltransferases. As expected, P5 hexapeptide was found to significantly inhibit both α2,3-sialyltransferase (Fig. 6*A*) and α2,6-sialyltransferase (Fig. 6*B*).

Inhibition of NeuAc Expression in CHO Cells—To determine whether P5 hexapeptide could inhibit the expression of NeuAc *in vivo*, CHO cells were treated with P5 hexapeptide in normal complex medium, and cellular glycoproteins were analyzed by RCA-I lectin blotting. It has been generally known that RCA-I interacts with high affinity with Galβ1,4GlcNAc sequence in asialo forms of bi-, tri-, and tetra-antennary *N*-glycans containing terminal β-linked galactose residues (31) and also interacts weakly with mucin-type Galβ1,3GalNAc sequence in *O*-glycan structure (32, 33). The CHO cells treated with P5 hexapeptide showed a dose-dependent inhibition of NeuAc expression on cellular glycoproteins (Fig. 7*A*, *lanes 3–5*), and Me_2SO treatment as a control resulted in a similar extent of RCA-I binding as detected in intact cells (Fig. 7*A*, *lane 2*). The treatment of CHO cells with a control hexapeptide (NH_2-WRGGSG) showed no significant inhibition of NeuAc expression on cellular glycoproteins, and also this control hexapeptide was tested not to inhibit the ST3Gal I activity *in vitro* (data not shown). As shown in Fig. 7*A*, however, the level of inhibition might not be maximized under this treatment condition. This result is not surprising if we consider the efficiency of peptide delivery across the cell membranes (34–36). To further confirm these observations in detail, we synthesized a more lipophilic peptide, FITC-P5 hexapeptide by conjugation of FITC to the primary amino group of P5 hexapeptide and applied to CHO cells. We also synthesized FITC-conjugated control hexapeptide, FITC-WRGGSG. Before cell treatment, the inhibitory activity of FITC-P5 against ST3Gal I was evaluated as much as that of P5 hexapeptide, whereas FITC-WRGGSG did not inhibit the ST3Gal I activity at all *in vitro* (data not shown). As expected, FITC-P5 hexapeptide treatment resulted in much stronger RCA-I binding in dose-dependent fashion (Fig. 7*B*, *lanes 3–5*) than P5 hexapeptide treatment. Me_2SO-treated cell lysates showed a background signal of RCA-I binding as in the case of intact cell lysates (Fig. 7*B*, *lanes 1* and *2*). The treatment of CHO cells with FITC-WRGGSG showed no significant effect on the inhibition of NeuAc expression (data not shown). These data demonstrate that both P5 and FITC-P5 can substantially inhibit sialyltransferases *in vivo*, and the FITC conjugation may enhance a peptide delivery in CHO cells. During these peptide treatments of CHO cells, we observed that cell morphology or growth rate was not influenced by the peptides (data not shown). Next, to test the inhibitory efficacy of P5 hexapeptide in a short term treatment of several hours, we used the Chariot™ system, a commercially available liposome system for an efficient peptide delivery into cells. After treatment of P5 hexapeptide together with Chariot™ for 2 h according to the

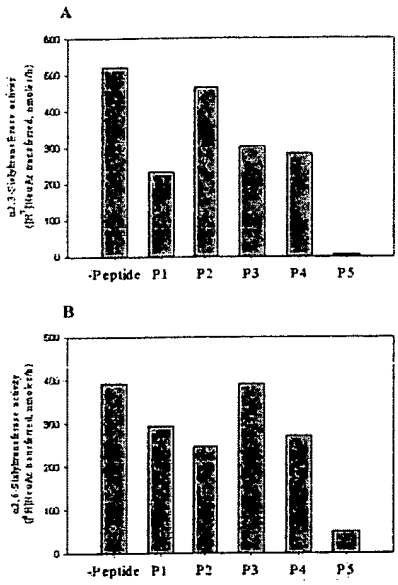

FIG. 6. **Inhibition of *N*-glycan-specific α2,3- and α2,6-sialyltransferase by five individual hexapeptides.** *A*, the inhibitory activities of five individual hexapeptides (P1–P5) against *N*-glycan-specific α2,3-sialyltransferase were analyzed using CMP-[^{3}H]NeuAc in the absence (−) and presence of hexapeptides as described under "Experimental Procedures." *B*, the inhibitory activities of five individual hexapeptides (P1–P5) against *N*-glycan-specific α2,6-sialyltransferase were comparatively analyzed.

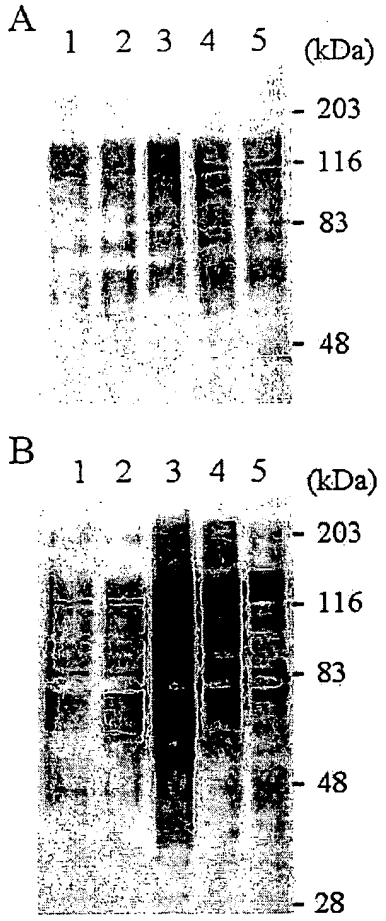

FIG. 7. **RCA-I lectin blot analysis of NeuAc expression in CHO cells treated with hexapeptide inhibitor.** *A*, CHO cells were cultured in α-minimal essential medium with 10% fetal bovine serum containing P5 hexapeptide at 10 μM (*lane 5*), 30 μM (*lane 4*), and 100 μM (*lane 3*) concentrations for 72 h, respectively. *B*, CHO cells were treated with FITC-P5 hexapeptide at 10 μM (*lane 5*), 30 μM (*lane 4*), and 100 μM (*lane 3*) concentrations for 72 h, respectively. The cells were harvested, and total glycoproteins were subjected to SDS-PAGE followed by RCA-I lectin blotting as described under "Experimental Procedures." Intact CHO cells (*A* and *B*, *lane 1*), cells treated with vehicle solvent of 0.5% Me_2SO (*A*, *lane 2*), and cells treated with 1.3% Me_2SO (*B*, *lane 2*) were analyzed as controls. Approximately 50 μg of proteins were applied to each lane of the gel.

manufacturer's protocol, we metabolically radiolabeled CHO cells with D-[6-^{3}H]$GlcNH_2$ for 8 h in the presence of P5 hexapeptide. We directly analyzed newly synthesized glycoproteins to examine whether the level of NeuAc expression was reduced. First, we analyzed the NeuAc expression on *O*-linked oligosaccharides by mild alkaline borohydride treatment followed by descending paper chromatography as previously described (28, 29). The majority of β-eliminated *O*-glycans from P5 hexapeptide-Chariot™-treated CHO cells contained nonsialylated GalNAcitol as a major *O*-glycan and nonsialylated core 1 structure (Galβ1,3GalNAcitol) as shown by the comigration with authentic standards (Fig. 8*D*). However, the β-eliminated *O*-glycans released from intact cells (Fig. 8*A*), Me_2SO-treated cells (Fig. 8*B*), and control hexapeptide-treated cells (Fig. 8*C*) were shown to be sialylated core 1 structure (mono- or disialylated Galβ1,3GalNAcitol) and sialylated GalNAcitol as major *O*-linked oligosaccharides, because desialylation of these samples by mild acid treatment generated Galβ1,3GalNAcitol and GalNAcitol (Fig. 8, *E–G*). These results demonstrate that P5 hexapeptide may inhibit not only ST3Gal I but also other *O*-glycan-specific sialyltransferases *in vivo*, such as ST6GalNAc I (37) and ST6GalNAc III (38). Our finding in the present study that CHO cells synthesize sialylated GalNAcitol structure in endogeneous glycoproteins seems to be quiet surprising, since no such glycan structure has been detected on any recombinant glycoproteins produced in CHO cells, although there is a report concerning the GalNAcitol structure produced in CHO cells (39). In a repeated experiment, a similar result was consistently obtained (data not shown), but the exact identity of this glycan structure was not further characterized. Second, we analyzed the expression level of NeuAc on *N*-linked oligosaccharides by QAE-Sephadex of anion exchange column chromatography (40). The majority of *N*-glycans released from the P5 hexapeptide-Chariot™-treated CHO cells were neutral oligosaccharides (Fig. 9, *B* and *D*); however, *N*-glycans released from intact cells, Me_2SO-treated cells, and control peptide-treated cells were shown to be mainly 2 and 3 negatively charged oligosaccharides, respectively (Fig. 9, *A*, *C*, *E*, and *F*). These negative charges were identified as NeuAc residues by mild acid treatment of negatively charged samples followed by rechromatography on QAE-Sephadex (Fig. 9). These results demonstrate that P5 hexapeptide can inhibit *N*-glycan-specific α2,3-sialyltransferase in CHO cells.

DISCUSSION

NeuAc residues expressed either on glycoproteins or glycolipids at the cell surface are known to exert important biological roles in cell-cell recognition of inflammation process (3) and especially in the metastatic potential of tumor cells (5, 6, 41). Therefore, it would be valuable to find out specific inhibitors of sialyltransferases for the therapeutic disease control. There have been a number of reports concerning the development of sialyltransferase inhibitors, such as CMP, CMP-NeuAc analogues (14), and NeuAc-neucleoside conjugates (16, 17, 61). Previous studies have shown that these compounds were evaluated as anti-metastatic agents in a variety of tumor models; however, their inhibition of sialyltransferases appears not to be promising *in vivo* (42). Recently, it has been reported that the

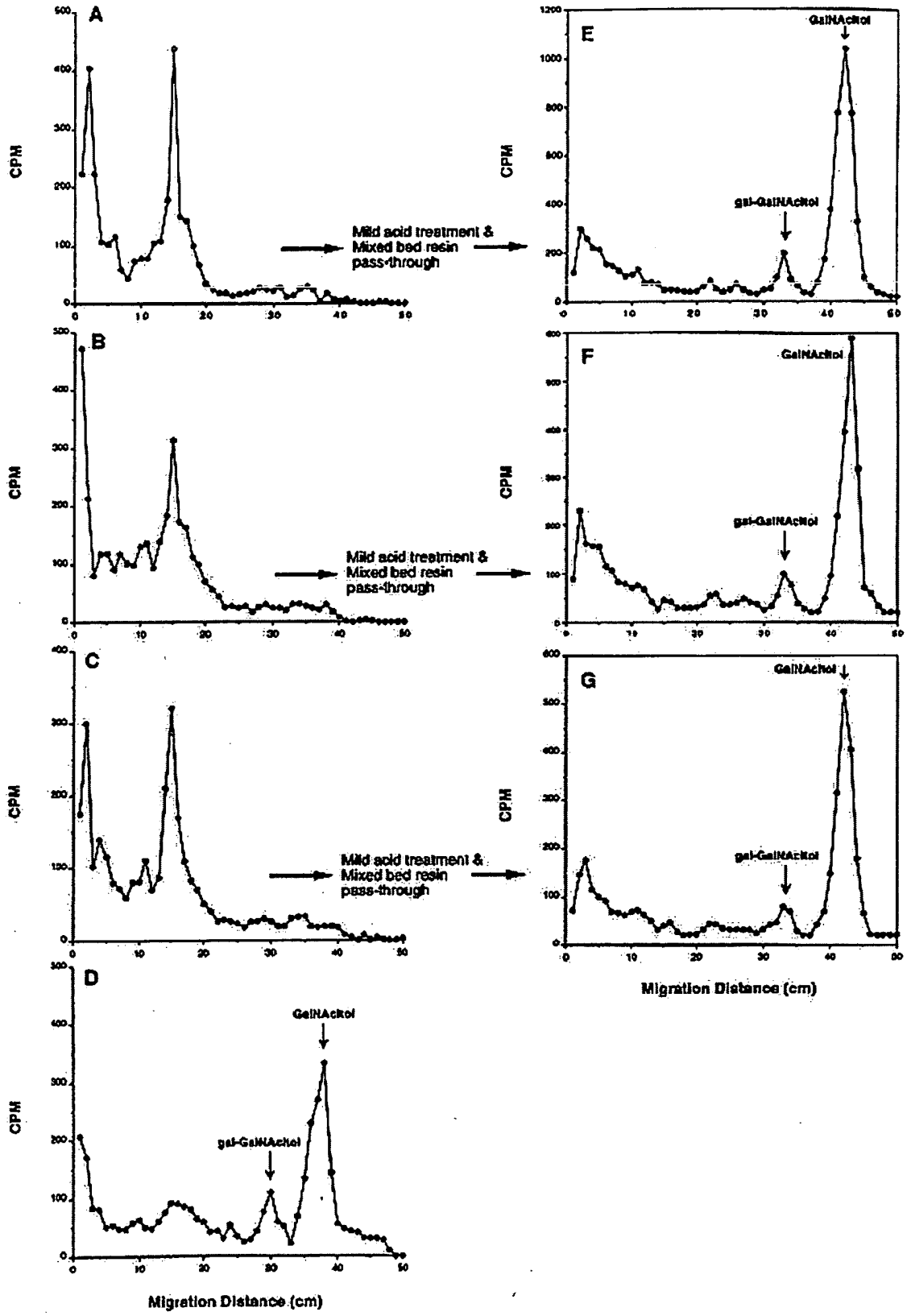

FIG. 8. **Analysis of the NeuAc expression on *O*-linked oligosaccharides of CHO cells by P5 hexapeptide treatment.** *A–D*, intact CHO cells (*A*) and cells treated with Chariot™ containing 0.5% Me_2SO (*B*), 50 μM control hexapeptide (*C*), and 50 μM P5 hexapeptide (*D*) were metabolically radiolabeled with D-[6-^{3}H]$GlcNH_2$ as described under "Experimental Procedures." The radiolabeled total glycoproteins were incubated with mild alkaline borohydride, and the released *O*-linked oligosaccharides were analyzed by descending paper chromatography in an 8:2:1 solvent system. *E–G*, the released *O*-glycans were treated with mild acid for desialylation. The desialylated materials from intact cells (*E*), 0.5% Me_2SO-treated cells (*F*), and control peptide-treated cells (*G*) were analyzed by descending paper chromatography as described under "Experimental Procedures." The *arrow* indicates the migration of authentic standards, [^{3}H]GalNAcitol and Galβ1,3[^{3}H]GalNAcitol.

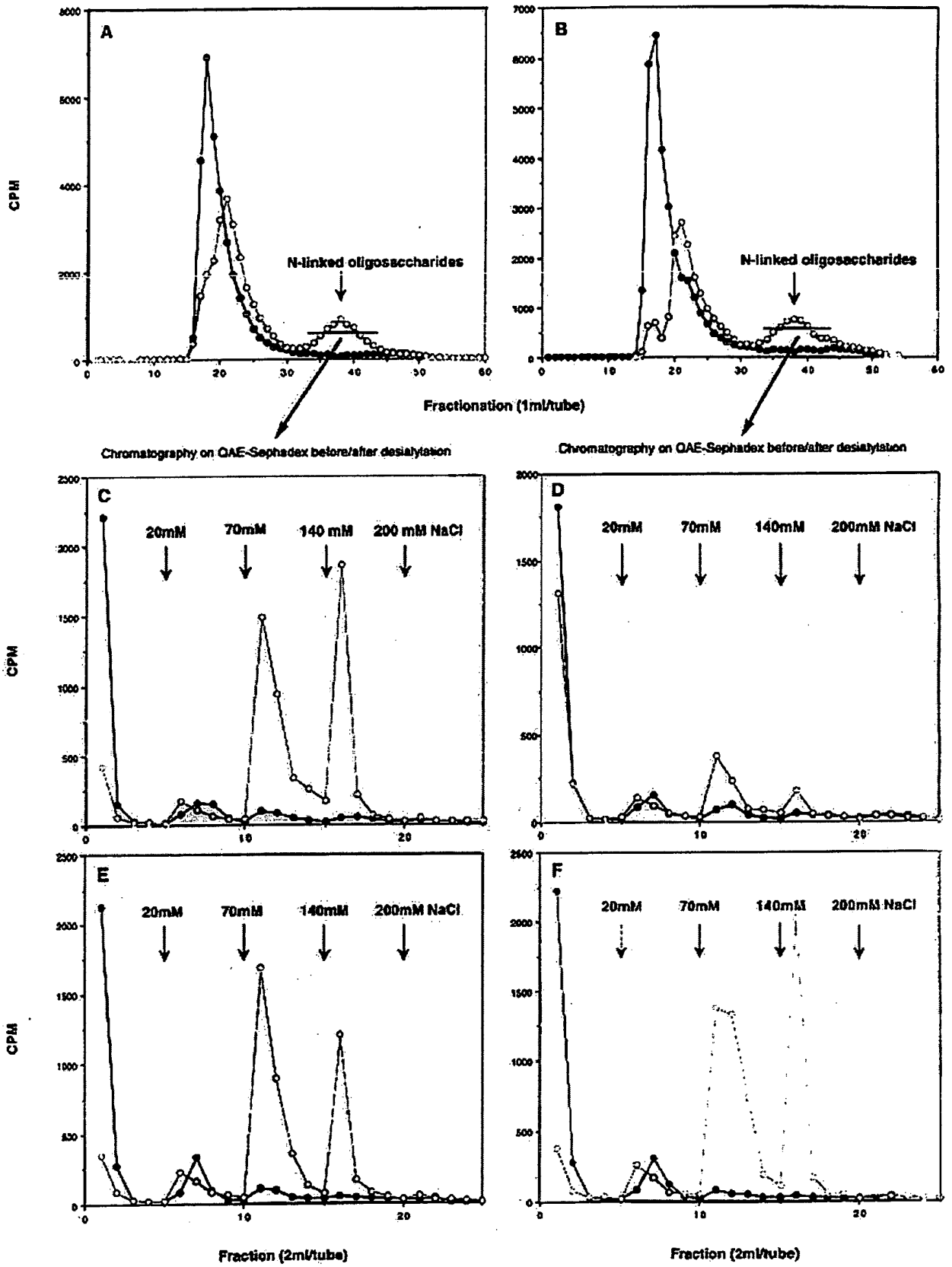

FIG. 9. **Charge analysis of *N*-linked oligosaccharides by QAE-Sephadex before and after P5 hexapeptide treatment.** *A–B*, total radiolabeled glycoproteins prepared from control CHO cells (*A*) and P5 hexapeptide-treated CHO cells (*B*) were applied on Sephadex G-50 before (*closed circle*) and after (*open circle*) *N*-glycanase treatment. *C–F*, the released *N*-linked oligosaccharides from intact cells (*C*), P5 hexapeptide-treated cells (*D*), 0.5% Me_2SO-treated cells (*E*), and control peptide-treated cells (*F*) were analyzed by QAE-Sephadex before (*open circle*) and after (*closed circle*) desialylation with mild acid treatment as described under "Experimental Procedures." The charged materials were eluted with increasing concentrations of NaCl as indicated by the *arrows*.

sialylation of core 1 *O*-glycan by ST3Gal I can compete with a formation of core 2 *O*-glycan in breast carcinoma cells (12). In addition, ST3Gal I enzyme has been shown to positively correlate with the expression of cancer-associated MUC1 in breast carcinomas (13).

In the present study, we expressed mouse cDNA encoding ST3Gal I in High Five™ cells, and we screened hexapeptide combinatorial library until the individual peptide sequence was obtained. To our knowledge, this is the first report to identify a hexapeptide inhibitor of ST3Gal I and to reveal that this hexapeptide functions as a generic inhibitor of a broad range of other sialyltransferases. In our expression system of High Five™ cells (43, 44), the level of secreted recombinant ST3Gal I was ~4–5 mg/liter, and protein A sequence linked to ST3Gal I was able to be efficiently utilized in a one-step purification of recombinant enzyme by IgG-Sepharose as shown in previous studies (45). Moreover, our data suggest that mammal-derived IgM signal sequence could efficiently be cleaved in High Five™ cells (Fig. 1*A*), and, based on tunicamycin treatment, the 1-kDa difference of molecular mass might be caused by other posttranslational modifications, such as *O*-glycosylation, phosphorylation, and sulfation (Fig. 2*B*). Recombinant ST3Gal I without *N*-glycosylation was found not to be secreted in the insect cell system, indicating that the exit of nonglycosylated ST3Gal I from the endoplasmic reticulum might be blocked as in mammalian cell system (46–48).

We finally obtained five different hexapeptides, and only P5 hexapeptide was found to significantly inhibit ST3Gal I sialyltransferase (Fig. 4*B*). The kinetic analysis of ST3Gal I inhibition showed that P5 can strongly compete with CMP-NeuAc binding to ST3Gal I enzyme. It is possible to consider some explanations for the molecular basis of an inhibitory mechanism. First, the P5 sequence may contain a structural similarity with CMP-NeuAc. This possibility suggests that the sequence of P5 hexapeptide can mimic a structural conformation of CMP-NeuAc. Alternatively, it is also feasible that P5 hexapeptide can partially mimic a portion of CMP-NeuAc. Recently, many studies have shown that the structural conformation of specific carbohydrate sequences can be sufficiently adopted by the specific peptide sequences (49–51). Furthermore, several studies on the carbohydrate-mimicking peptides have shown that aromatic amino acids in the peptide sequence could function as a critical factor to reflect the specific carbohydrate conformations (52–55). Second, the P5 hexapeptide sequence might be able to possess a similar conformation of the CMP-NeuAc binding pocket in ST3Gal I enzyme. It is now known that the sialylmotif L in mammalian sialyltransferases is highly conserved and involved in CMP-NeuAc binding (8). Interestingly, the first two amino acids of P5 hexapeptide sequence are well conserved in the sialylmotif L of mammalian sialyltransferases. Therefore, we can hypothesize that these two amino acid residues, Gly and Asn, in the sialylmotif L may provide a likely contact point to the CMP-NeuAc. At present, the molecular details of how the P5 hexapeptide sequence interacts with ST3Gal I enzyme are under investigation. Based on the present data of kinetic analysis, our study is highly suggestive that P5 hexapeptide can inhibit a broad range of sialyltransferases, such as *N*-/*O*-glycan-transferring sialyltransferases (8) as well as glycolipid-specific sialyltransferases (56). Therefore, we tested this idea and confirmed that the P5 hexapeptide inhibited both *N*-glycan-specific α2,3- and α2,6-sialyltransferase regardless of its linkage specificity (Fig. 6).

To test the inhibition of sialyltransferase *in vivo*, we treated CHO cells with P5 and FITC-P5, a derivative of P5. FITC-P5 was synthesized through a conjugation of FITC onto primary amine at the NH_2 terminus of the P5 hexapeptide sequence to increase peptide lipophilicity. The inhibitory activity of FITC-P5 *in vitro* was tested to be fully active as much as P5, indicating that the modification of the primary amino group of P5 must not be crucial for ST3Gal I inhibition.

In our inhibition assay *in vivo* using RCA-I blotting, both P5 and FITC-P5 peptide can inhibit the expression of NeuAc in CHO cells. Moreover, FITC-P5 appears to have much stronger inhibition than P5, indicating that peptide delivery might be important for an efficient inhibition of sialyltransferases *in vivo*. The smeared pattern of RCA-I blotting, which covers 30–200-kDa glycoproteins indicates that the whole range of glycoproteins might be affected regardless of *N*- and *O*-linked glycans (Fig. 7). It should be noted that the significant detection of nonsialylated and terminally galactosylated glycoproteins by RCA-I blotting requires a sufficient accumulation of newly synthesized glycoproteins and also a complete turnover of presynthesized glycoproteins during the peptide treatment of CHO cells. Considering the fact that the half-life ($t_{1/2}$) of membrane glycoproteins, in general, could be longer than 20–24 h, we maintained cells up to 72 h in the presence of P5 hexapeptide to maximize the detection of RCA-I blotting (Fig. 7). We also tested the inhibitory efficacy of P5 in a short term treatment of CHO cells for several hours. In this case, we treated cells with P5 hexapeptide encapsulated by Chariot™ liposome to facilitate a peptide delivery into cells. The structural analyses of newly synthesized *N*- and *O*-linked oligosaccharides showed that P5 substantially inhibited more than 90% of glycoprotein sialylation in CHO cells. Furthermore, our results demonstrate that the expression of NeuAc on both *N*- and *O*-linked oligosaccharides was significantly reduced within a 10-h treatment of P5 hexapeptide (Figs. 8 and 9). Our present results further confirm that the P5 hexapeptide may inhibit ST3Gal I as well as other sialyltransferases such as ST6GalNAc I (37), ST6GalNAc III (38), and ST3Gal III (57) *in vivo*. Unexpectedly, we found that the endogenous *O*-linked oligosaccharides synthesized in CHO cells contained sialylated GalNAc structure, although the exact identity of *O*-linked sialylated GalNAc structure needs to be further characterized. Until now, it has been reported that most recombinant glycoproteins produced in CHO cells were identified to contain sialylated core 1 structure as a major *O*-linked oligosaccharides (58–60) and, in some cases, to contain Tn structure (39).

In conclusion, these results suggest that P5 hexapeptide, NH_2-GNWWWW, may function as a generic inhibitor of a broad range of *N*- and *O*-glycan-specific sialyltransferases as predicted in our kinetic analysis. Further studies are needed to define the molecular basis of how P5 hexapeptide interacts with sialyltransferases *in vivo*. The inhibitory activity of P5 hexapeptide against a broad spectrum of sialyltransferases may be applied as a therapeutic tool to treat human diseases, such as inflammation, cancer metastasis, and viral infection.

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